AD-A146 141 A BIODEGRADABLE PROTEOLIPID BONE REPAIR COMPOSITE(U) 1/1
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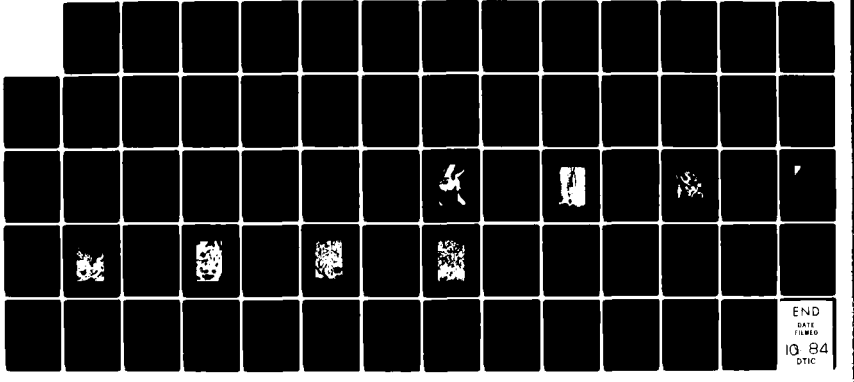

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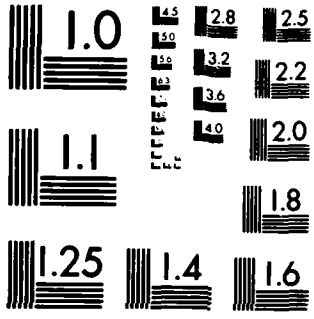

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A Biodegradable Proteolipid Bone Repair Composite

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A Biodegradable Proteolipid

Bone Repair Composite

ABSTRACT

A synthetic proteolipid was combined with a biodegradable copolymer (50:50 poly(L)-lactide co-glycolide) and inserted into defects prepared in the tibias of rats. At 3, 7, 14, 21, 28, and 42 days, animals were sacrificed and the implant sites were prepared for histomorphometric evaluation. Control defects, defects filled with copolymer alone, and defects filled with copolymer combined with proteolipid were analyzed with a Zeiss Image Analysis System with Osteoplan™ version 4.1.

A two-way analysis of variance revealed more reparative bony elements in the defects filled with copolymer-proteolipid at an earlier period of time than either the copolymer alone or control sites. The copolymer-proteolipid implant may prove to be an alternative to the agents commonly used for osseous repair.

INTRODUCTION

Many different types of materials have been used for the purpose of repairing, replacing, or augmenting bone. At the present time, autografts are the favored modality of treatment used by orthopedic and maxillofacial surgeons.[1] Unfortunately, there are numerous disadvantages associated with autogenous grafting, such as (1) failure rates ranging from 13% to 30%, (2) inability to recover sufficient autogenous bone to meet the needs of the host, (3) technical inconvenience, and (4) trauma to the patient as a consequence of a second surgical procedure.[2,3] Bone allografts, alloimplants, demineralized bone, and collagen gels have been used in attempts to rectify this problem, often with mixed results.[3-8] The use of resorbable and nonresorbable ceramics for bone repair or augmentation also has been described extensively.[9,10] This class of agents, while possessing limited orthopedic utility, may be useful in the treatment of certain intraosseous periodontal defects.

Biopolymers known as poly-alpha-hydroxy acids (a class of polyesters) have garnered considerable attention in the medical and dental fields in the past ten to fifteen years. The poly-alpha-hydroxy acids known as polyglycolic acid (PGA) and polylactic acid (PLA) were initially formulated and described as biodegradable suture materials.[11-13] They are commercially available as Dexon® and Vicryl®. Different formulations of the PLA and PGA also have

been used experimentally for osseous repair and reconstructive procedures.[14-18]

Considerable attention has been focused on the type II matrix vesicle for initiating calcification.[19-21] Intensive investigation of the structure of the matrix vesicle has revealed that its trilaminar membrane possesses a high content of acidic phospholipid.[22,23] It has been shown that a protein-acidic phospholipid complex similar to that of the matrix vesicle can induce hydroxyapatite formation both in vitro and in vivo.[24-26] The formulation of the protein-acidic phospholipid complex is of a paste-like consistency that is unsatisfactory for most types of bony wound applications, such as when rigid fixation is required or when expansive discontinuity defects must be repaired.[26] It was the purpose of this study, therefore, to develop a composite bone repair material that would have utilitarian application. The combination of the biodegradable polyesters of PLA and PGA with a protein-acidic phospholipid component was conceived for such a purpose.

MATERIALS AND METHODS

Preparation of the Implant Material

The acidic phospholipid, diphosphoinositide (phosphatidyl inositol 4,5 diphosphate), was combined with a commercially available lysozyme (mucopeptide - N - acetylmuramoylhydrolase) in a volume ratio of 2:1, producing a proteolipid complex (DPI-L)[26]

(Figure 1). A commercially synthesized copolymer, 50:50 poly(L)-lactide co-glycolide, (50:50 PLA:PGA), with a weight-average molecular weight of 80,000 Daltons was solubilized in methylene chloride at a 1:12.5 weight:volume ratio. The 50:50 PLA:PGA copolymer was precipitated with anhydrous methanol (1:1 volume ratio) and forced into a 2.0mm X 1.25mm Teflon® mold to produce 140 implants. Copolymer-proteolipid implants were prepared in a similar manner by combining the precipitated copolymer with DPI-L (1% by weight proteolipid to copolymer). Following lyophilization (50°C, 5 millitorr, 48 hrs) to remove residual methylene chloride, all implants were sterilized in ethylene oxide (30°C, 4-5 psi, 6 hrs) and weighed (final weights equaled 0.71 $\pm$ 0.10 gm) (Figure 2).

Preparation of Experimental Animals

One hundred eighty Walter Reed strain of rats (random males and females, average weight 200-300gm) were anesthetized with intraperitoneal sodium pentobarbital, USP, at a dosage of 3-5mg per 100mg of body weight. The 180 rats were divided into three groups of 60 each: Group A received copolymer-DPI-L implants, Group B received copolymer implants, and Group C served as controls.

Surgical Procedure

The diaphyses of the right and left tibia were selected as the implant and control sites. Areas over the diaphyses were shaved and scrubbed with povidone iodine, N.F. (Betadine®) for 5 minutes. A 1cm incision was made on the anterolateral surface of each tibia

and following reflection of soft tissues, a circular defect (1.95mm O.D.) was prepared through the cortical plate with a bone trephine and sterile water coolant (Figure 3). Copolymer-proteolipid implants (120 in number) were inserted into each tibia of 60 experimental animals; the second group received 120 copolymer implants; the 60 controls received no implants (Table I). All surgical sites were closed in layers with 3-0 Dexon®. At 3, 7, 14, 21, 28, and 42 days, ten animals from each of the three groups were sacrificed with intraperitoneal sodium pentobarbital, USP, and control and experimental sites were retrieved along with a segment of contiguous surrounding bone (Figure 4).

Analysis of the Specimens

Specimens were embedded in methylmethacrylate, sectioned at five microns, and stained with Goldner trichrome stain to facilitate histomorphometric analysis. Each specimen was analyzed with a Zeiss Image Analysis System with Osteoplan™ (version 4.1) to derive histomophometric data (Tables II and III).

DATA ANALYSIS

Data from the analyses of the same variable (i.e., bony trabecula) from the same treatment and temporal groups were combined, yielding a pooled mean value based upon 2,000 measurements (Table II). A standard deviation and a standard error of the mean were computed for each set of the 2,000 measurements for each value.[27,28] The pooled mean for each variable (based upon 2,000 fields)

plus and minus its standard error of the mean was then used to construct a histogram. A trio of sets (i.e., A_3, B_3, C_3) representing treatment and temporal groups were arranged along the abscissa and the corresponding units or percentage appeared along the ordinate (Histograms I through IX).

A two-way analysis-of-variance computer program was used to examine and to test the effects of (1) the treatment groups (differences between the three treatment groups analyzed over the six time periods) and (2) time periods (differences between the six time periods averaged over the three treatment periods). Effects having an observed significance level (p-value) equal to or less than 5% (based on the appropriate F-test value which was derived by performing the two-way analysis of variance) were considered to be statistically significant.

RESULTS

Statistical and Histomorphometric

The statistical evaluation based upon the analyses of 180,000 possible histomorphometric measurements is summarized in Table 1. Histograms I-IX represent samples of the histograms derived from the data for each of the variables that were analyzed with the Image Analysis System.

Histological

The overall trends in healing frequently displayed only subtle visual differences when viewed histologically. Photomicrographs of

selected stages of repair can be observed in Figures 5-10.

All treatment groups displayed the typical patterns of osseous wound healing. There was often equivocal evidence that histologically evaluated wound healing might have been superior for one type of treatment class but not for another; however, when histomorphometric assessments and statistical analyses were performed, contrasts were extracted that frequently proved to be significantly different.

Histological evaluation seemed to indicate that the patterns of healing consisted of more reparative elements in the copolymer-proteolipid treated sites at an earlier period in time than in any of the plain copolymer or control sites. Furthermore, plain copolymer treated wounds appeared to possess more of the elements of osseous repair at the same time periods than did the controls. It did not appear that the presence of the composite implant material deterred bony healing, which occurred centripetally. There was no callus formation and no evidence of an adverse inflammatory response engendered from the two implant treated groups.

DISCUSSION

Materials such as bone grafts and implants, collagen gels, ceramics, bone derivatives, and biopolymers are some of the many agents which have been employed by orthopedic and maxillofacial surgeons for initiating osseous repair or for replacing bone. Failure to achieve beneficial results with these materials has not

been necessarily a consequence of imprudence, but rather due to deficiencies inherent in the repair and replacement agents. A combination of the biopolymers PLA and PGA in conjunction with the particular proteolipid described, appears to offer considerable promise as an alternative to the more common, conventional materials.

It has been mentioned that the proteolipid complex establishes a unique chemical environment conducive to calcium and phosphate precipitation, nucleation, and subsequent crystal growth.[26] Moreover, the implication is that the locally introduced proteolipid was tantamount to surrogate extracellular matrix vesicles, the structure whose limiting membrane is heavily endowed with an acidic phospholipid component. The extremely critical function of matrix vesicles in the calcification scheme has been described at length.[21,29,30]

The breakdown of the biopolymers of PLA and PGA occurs by nonspecific hydrolytic scission that results in the generation of lactic acid and glycolic acid residues.[31-33] The lactic acid becomes incorporated in the TCA cycle and is excreted by the lungs as carbon dioxide and water. Glycolic acid dimers, trimers, etc., are enzymatically degraded by esterases and carboxy peptidases[34] and are converted to monomers of glycolic acid which either can be excreted in the urine or enzymatically converted by glycolate oxidase to glyoxylate.[31] This moiety reacts with glycine trans-

aminase and the glycine that is produced can be used for synthesis of serine, which can be employed in the TCA cycle after transformation into pyruvate.[35,36]

The positive bone healing response engendered in experimental animals from the copolymer of PLA and PGA may be a consequence of several factors.[37] The linear polyester macromolecular structure could act as a matrix, trellis, or foundation upon which bony reparative elements may be consolidated. Furthermore, a possible consequence of the degradation of the copolymer could be that the pH of the local environment was altered and the potential inhibitors to calcification (proteoglycans, glycosaminoglycans) were debilitated and rendered ineffectual. The pH changes and the organic monomeric acid residues interaction with host organic matrix could function as a mechanism promoting release from the matrix of certain polypeptides, such as bone morphogenetic protein and human skeletal growth factor.[38] These factors have been speclated as being agents capable of increasing both osteoblast progenitor cell proliferation and subsequent bone formation rate.

The material described may benefit the orthopedic and maxillofacial surgeon, because it is tissue tolerant and may be made readily available as an off-the-shelf type of bone substitute. Additionally, it is osteoconductive and may be conveniently shaped into a wide variety of geometries at the time of surgery.

CONCLUSION

Evaluation of the hypothesis that a copolymer of PLA and PGA combined with a protein-acidic phospholipid could facilitate bony wound repair was accomplished by histochemical and computer-assisted histomorphometric techniques. The results of the evaluation indicate that the bone repair material was successful at stimulating the early phases of osseous repair and suggest that it may be an alternative to the agents commonly employed for bone repair and reconstruction.

MILITARY DISCLAIMER

Commercial materials and equipment are identified in this report to specify the investigative procedures. Such identification does not imply recommendation or endorsement or that the materials and equipment are necessarily the best available for the purpose. Furthermore, the opinions expressed herein are those of the authors and are not to be construed as those of the US Army Medical Department.

ANIMAL STATEMENT

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care, of the Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council.

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LEGENDS FOR FIGURES

Figure 1 Implant Preparation

Figure 2 Implant Preparation

Figure 3 Anterolateral surface of tibia showing implant site

Figure 4 Implant site at sacrifice

Figure 5 Photomicrograph of copolymer-proteolipid (Group A) treated wound site at three days. Bone trabeculae (*) haphazardly oriented and rimmed with osteoid (Δ) and osteoblasts (→) (40X)

Figure 6 Photomicrograph of copolymer (Group B) treated wound site at three days. Area where osteoid (Δ) being deposited and less calcified material present than for Group A (40X)

Figure 7 Photomicrograph of control (Group C) wound site at three days. Swirling pattern of fibrous connective tissue with numerous fibroblasts and occasional osteoid and bony trabeculae formation (40X)

Figure 8 Photomicrograph of Group A at twenty-eight days with numerous trabeculae that are coalescing (40X)

Figure 9 Photomicrograph of Group B at twenty-eight days demonstrating an area of active repair adjacent to the intact cortex (16X)

Figure 10 Photomicrograph of Group C at twenty-eight days displaying normal elements of wound repair, such as trabeculae, osteoid, and osteoblasts (40X)

LEGENDS FOR HISTOGRAMS

Histogram	I	Volumetric Density of Marrow (VV-MAR)
Histogram	II	Fraction of Trabecular Surface Exhibiting Resorptive Lacunae (L-TOT%)
Histogram	III	Mean Osteoid Thickness (TM-OS)
Histogram	IV	Volumetric Density of Bone (VV)
Histogram	V	Osteoclast Index (OCI)
Histogram	VI	Fraction of Total Trabecular Surface Covered by Osteoid (OB%)
Histogram	VII	Volumetric Density of Osteoid (VV-OS)
Histogram	VIII	Osteoblast Index (OBI)
Histogram	IX	Mean Trabecular Diameter (D-TRAB)

TABLE I

Organization of Treatment and Temporal Groups

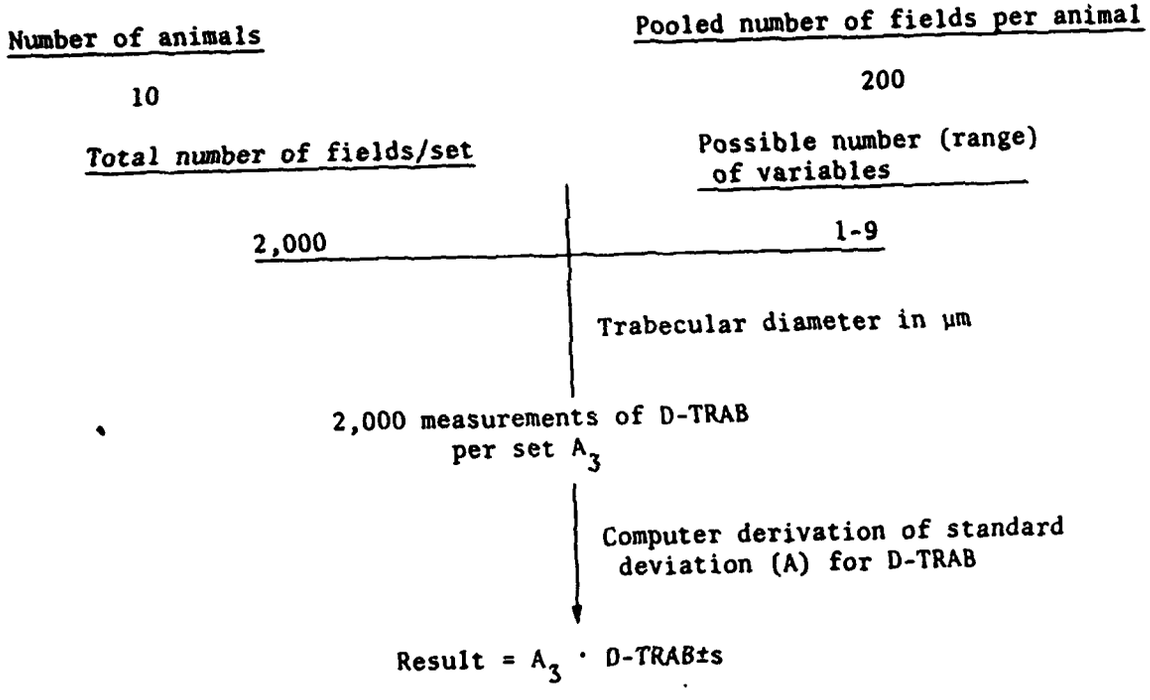
Treatment group (Each treatment group had 10 animals per temporal group)	A (50:50 PLA: PGA-DPI-L)	B 50:50 PLA: PGA	C Control
Temporal group (Number of days post-treatment that animals were sacrificed)			
3	10	10	10
7	10	10	10
14	10	10	10
21	10	10	10
28	10	10	10
42	10	10	10
Total number of animals per treatment group	60	60	60

PLA:PGA = Polylactic acid: Polyglycolic acid

DPI-L = Diphosphoinositide - Lysozyme

TABLE II

Organization of Data for the Variable Trabecular Diameter (D-TRAB) from Treatment Group A, Temporal Group 3 Days



Key:

T = Temporal group (3, 7, 14, etc.) in days

Tx = Treatment group (A, B, C)

Set = (Tx) (T) = A_3, B_3, C_3, A_7, etc.

s = Standard deviation

TABLE III

Example of Possible Number of Variables from Treatment Groups A, B, and C

Temporal groups (T) in days	3	7	14	21	28	42	
Pooled number of fields	200	200	200	200	200	200	
Number of animals per T	10	10	10	10	10	10	
Total number of fields	2,000	2,000	2,000	2,000	2,000	2,000	
						Grand total	12,000

Explanation:

1. There were ten animals per temporal group (T).

2. There were 200 fields measured per animal; therefore, 2,000 fields were measured per T (200 X 10 = 2,000).

3. The possible range of derived variables was from one to nine with a mean of five; therefore, 10,000 pieces of information could be computed per T (2,000 X 5. = 10,000).

4. The term set can be defined as a treatment group (Tx) at a particular time (that is, a T of 3, 7, 14, 21, 28, or 42).

5. A grand total (average) of 180,000 measured values could, therefore, be derived (10,000 X 18 = 180,000).

TABLE IV

Results of the Two-Way Analysis of Variance of Sum of Squares

	Time derived values		Sum of squares		Treatment derived values	
	F-test	p	Treatment	Time	F-test	p
Variable ▲						
VV	65.08●●		24,782	192,311	21.32●	
Treatment contrasts ▲▲						
A vs. B		**				**
A vs. C		**				**
B vs. C		**				**
D-TRAB	179.93●●		2,322	38,603	27.02●	
A vs. B		**				**
A vs. C		**				**
B vs. C		**				**
TH-OS	127.79●●		205	1,958	33.34●	
A vs. B		**				*
A vs. C		**				**
B vs. C		**				**
VV-OS	85.03●●		10.2	205.7	10.21●	
A vs. B		**				
A vs. C		**				**
A vs. C		**				*

TABLE IV (Con't.)

Results of the Two-Way Analysis of Variance of Sum of Squares

Variable ▲	Time derived values F-test	p	Sum of squares Treatment	Time	Treatment derived values F-test	p
OBI	53.99●●		5,706	123,364	6.85●	
Treatment contrasts ▲▲						
A vs. B		**				**
A vs. C		**				*
B vs. C		**				
OB%	53.99●●		157	4,480	6.85●	
A vs. B		**				**
A vs. C		**				*
B vs. C		**				
OCI	7.55●●		6.8	1,857.3	2.43●	
A vs. B		**				
A vs. C		**				
B vs. C		**				
L-TOT%	247.51●●		0.51	280.22	1.02●	
A vs. B		**				
A vs. C		**				
B vs. C		**				

TABLE IV (Con't.)

Results of the Two-Way Analysis of Variance of Sum of Squares

Variable ▲	Time derived values F-test	Time derived values p	Sum of Squares Treatment	Sum of Squares Time	Treatment derived values F-test	Treatment derived values p
VV-MAR	495.59●●		9,815	512,756	23.73●	
Treatment contrasts ▲▲						
A vs. B		**				**
A vs. C		**				**
B vs. C		*				**

Key: ▲ = Variables described in section III. J.

** = p <0.01

* = p <0.05

●● = F confidence level 99%

● = F confidence level 95%

▲▲ A = Copolymer plus proteolipid

B = Copolymer

C = Control

(VV) = Volumetric density of bone

(D-TRAB) = Mean trabecular diameter

(TH-OS) = Mean width of osteoid

(VV-OS) = Volumetric density of osteoid

(OBI) = Osteoblast index

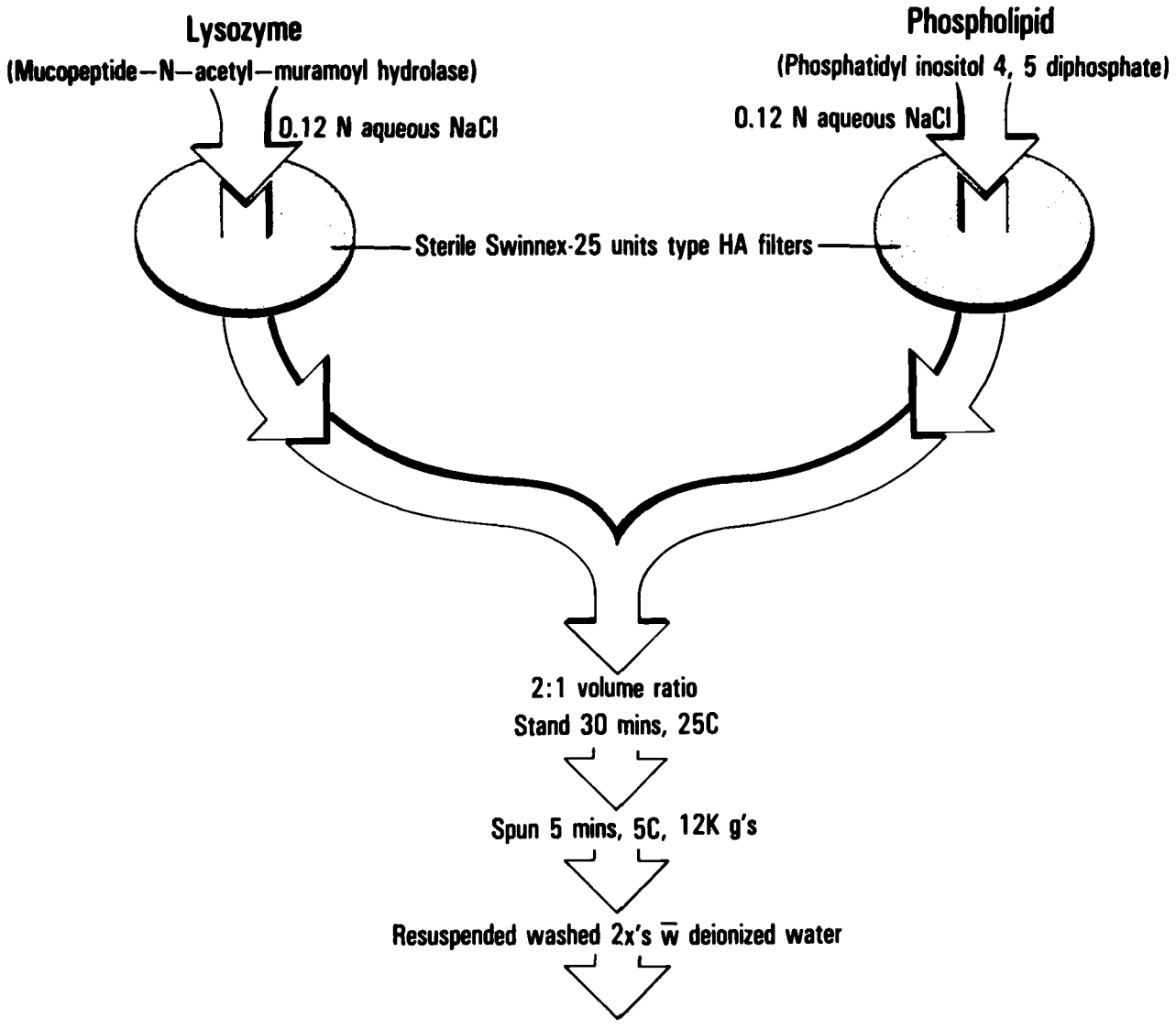
(OB%) = Fraction of total trabecular surface covered by osteoid

(OCI) = Osteoclast index

(L-TOT%) = Fraction of trabecular surface exhibiting resorptive lacunae

(VV-MAR) = Volumetric density of marrow

IMPLANT PREPARATION



67075-Y

IMPLANT PREPARATION

50:50 PLA:PGA
(50:50 poly (L (–) lactide co-glycolide))

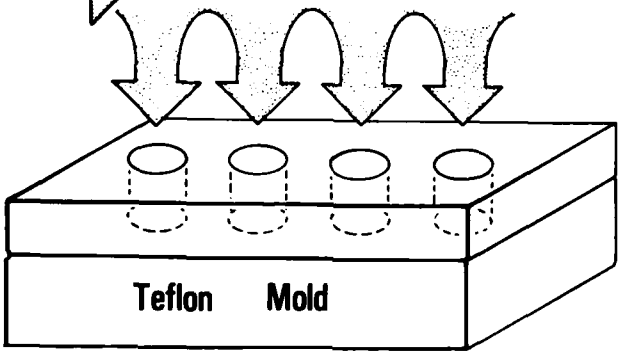
Methylene Chloride
1:12.5
weight volume

Suspension

Anhydrous Methanol 1:1

50 mg
DPI–L

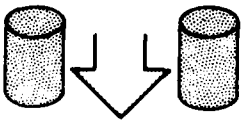
Gelatinous Mass: Kneaded 5 mins



Lypholized

50° C 5 millitorr
48 Hrs.

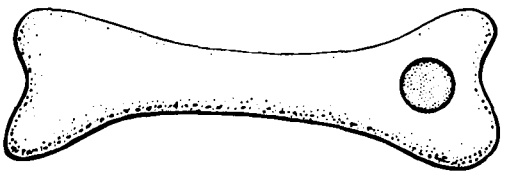
Implant Plugs:
2.0 mm x 1.25 mm



Stored in dessicator
4–5 psi
30° C
6 hrs

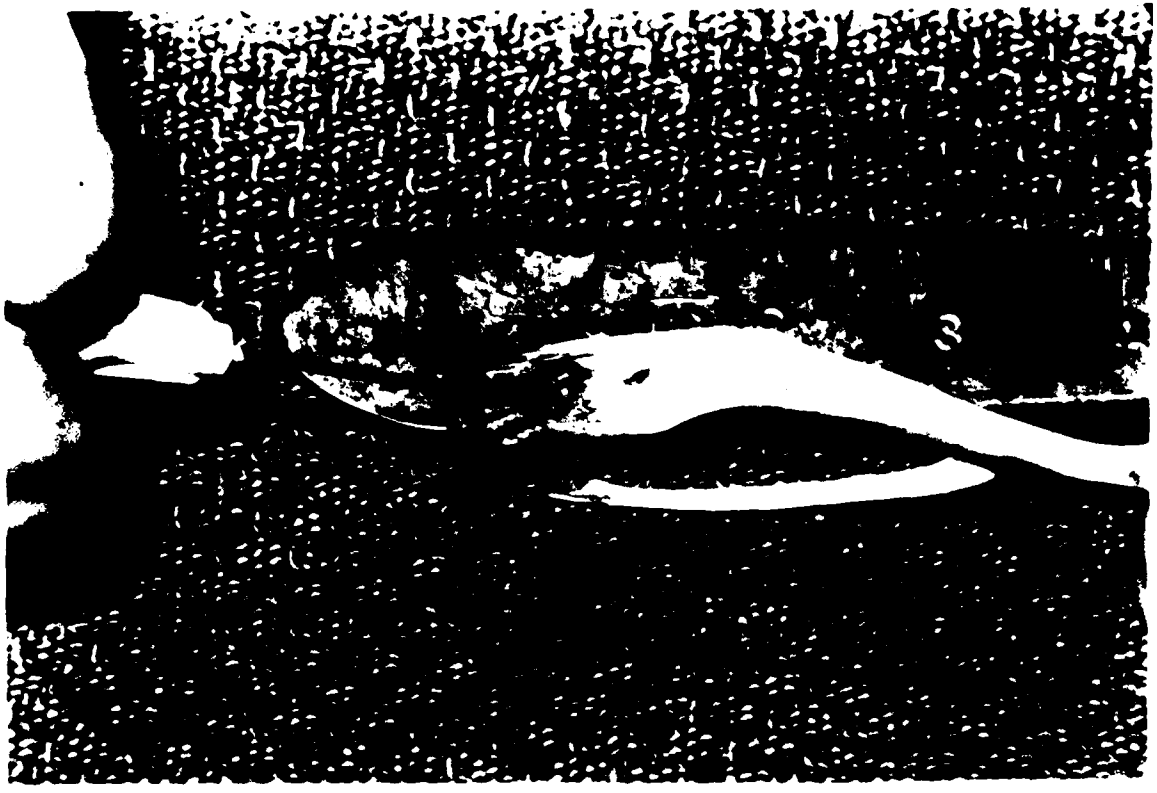
Sterilized: Ethylene Oxide

Implant plug into
diaphysis of tibia of rat

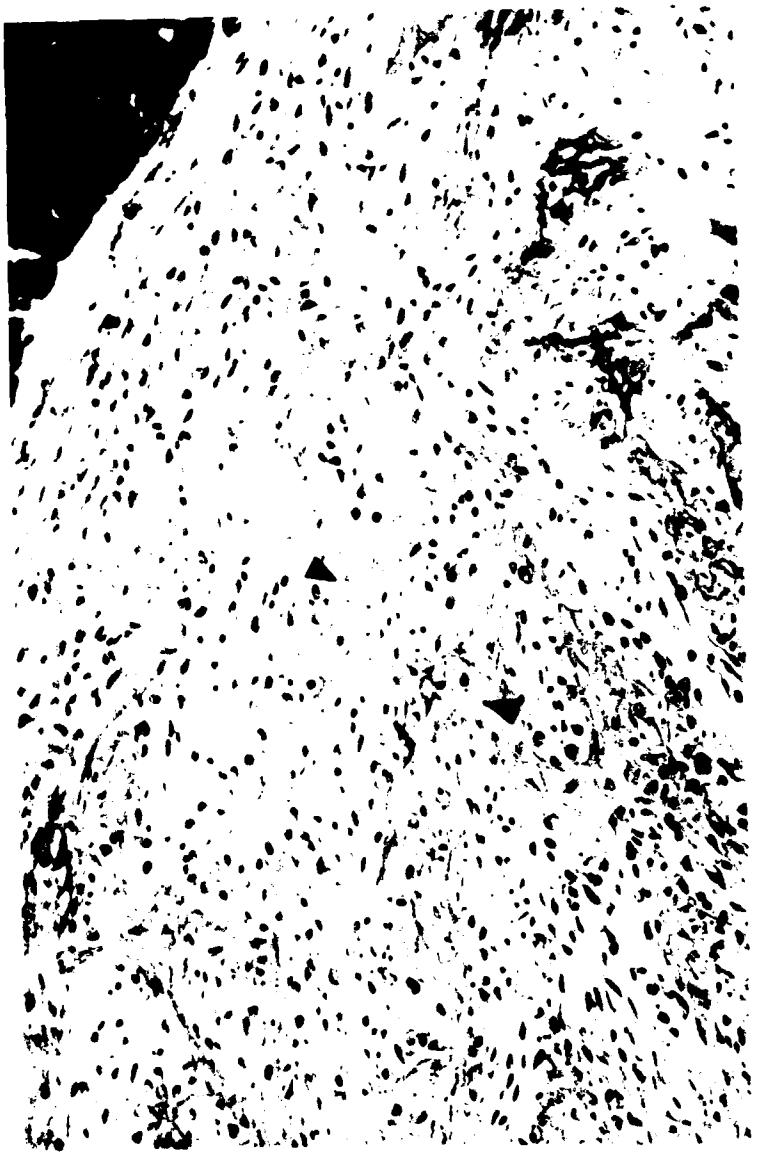


67015-1

72528



72528

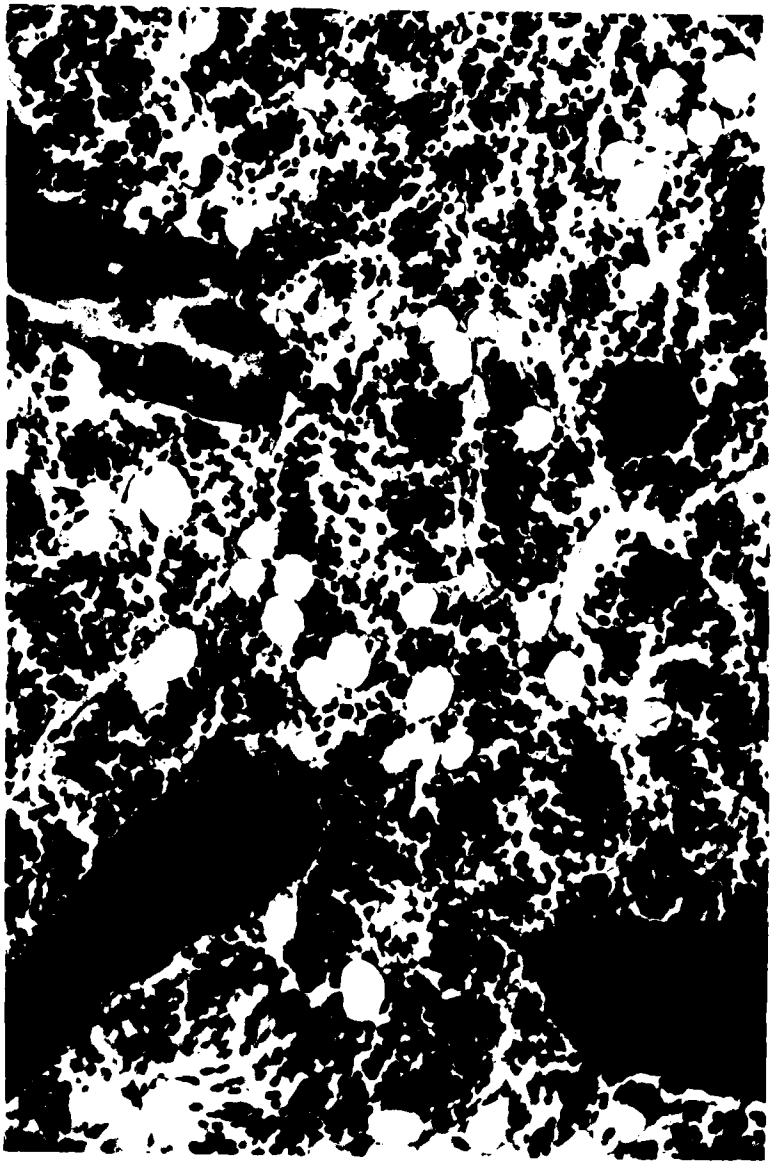


70574

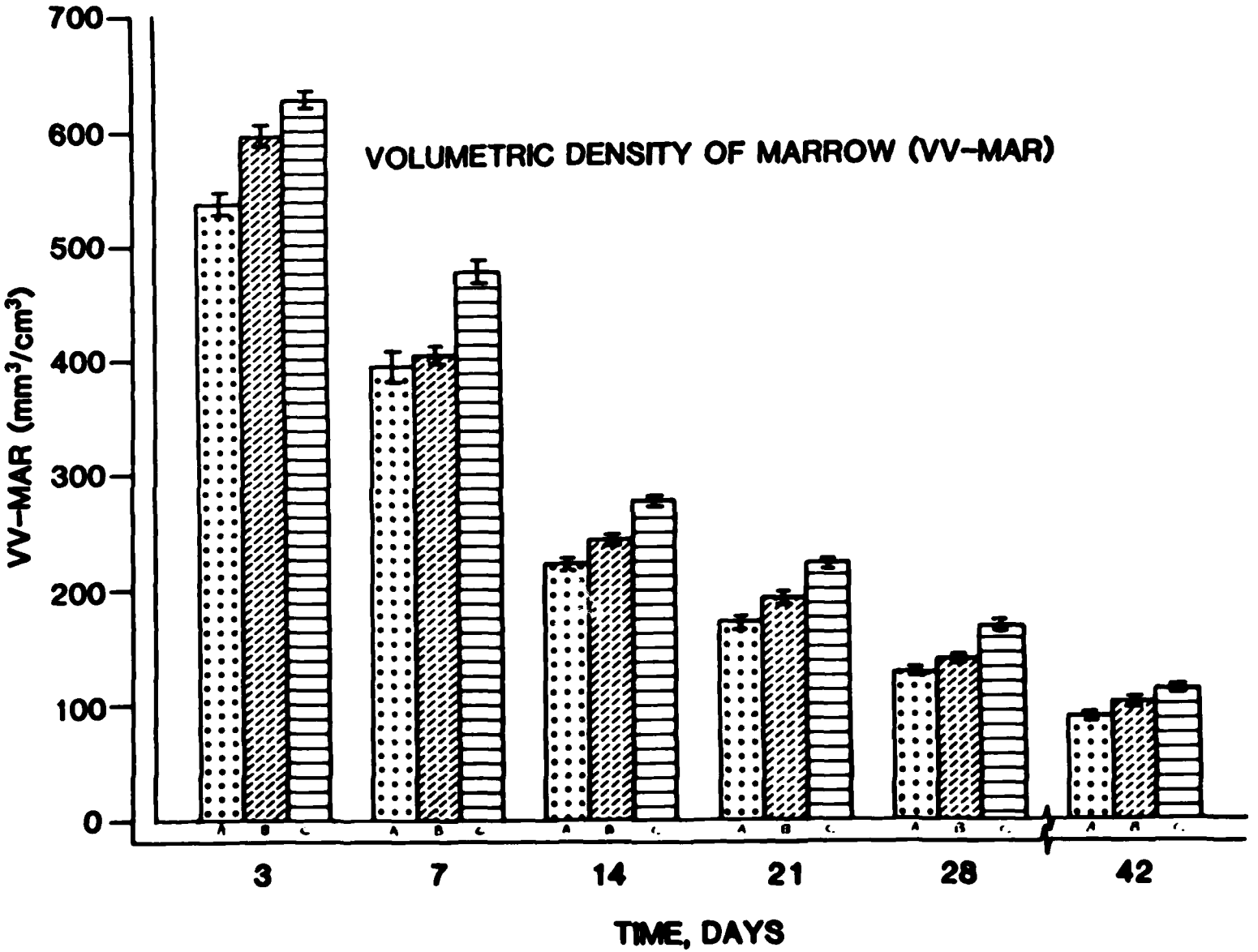
70576

70576

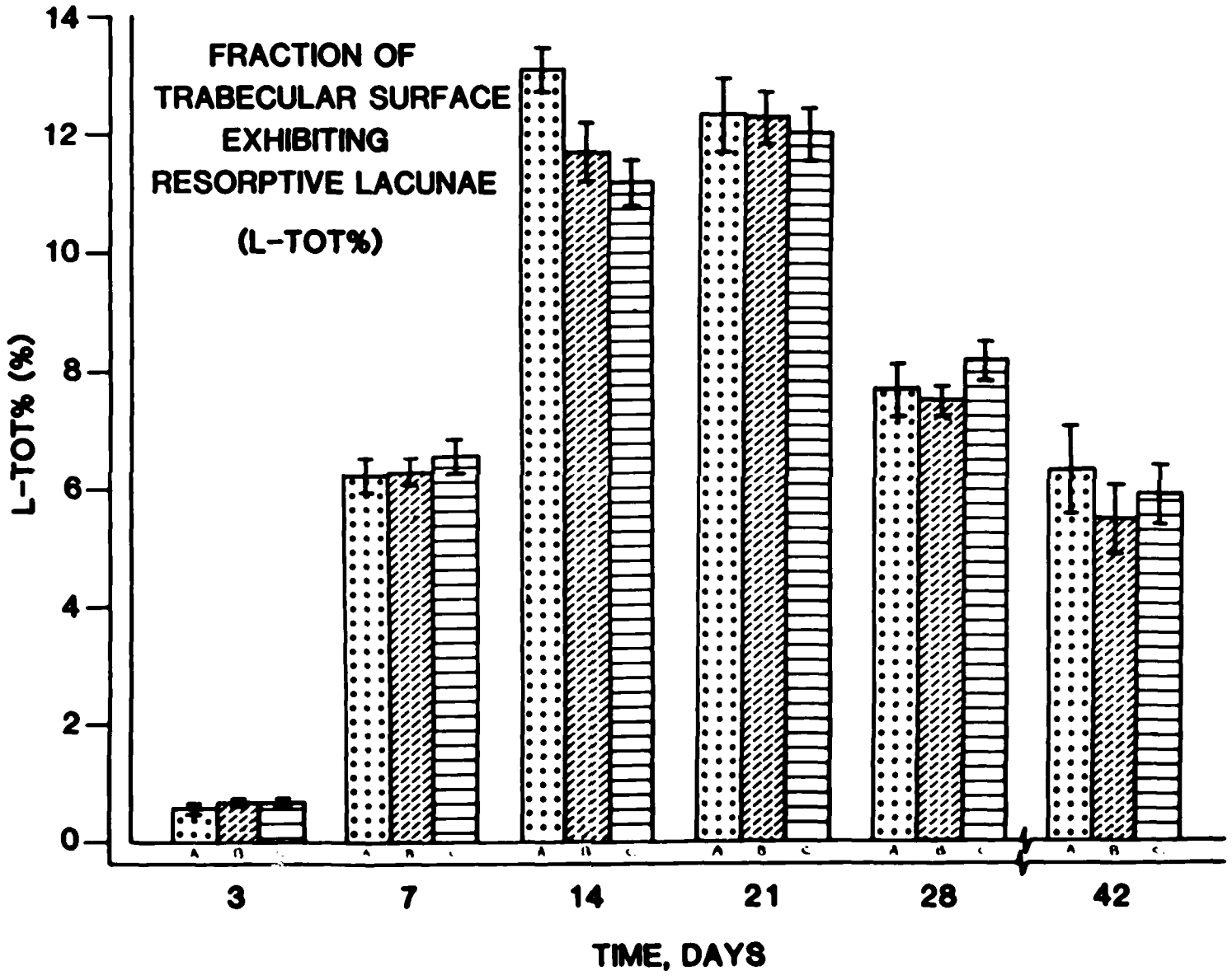
70576



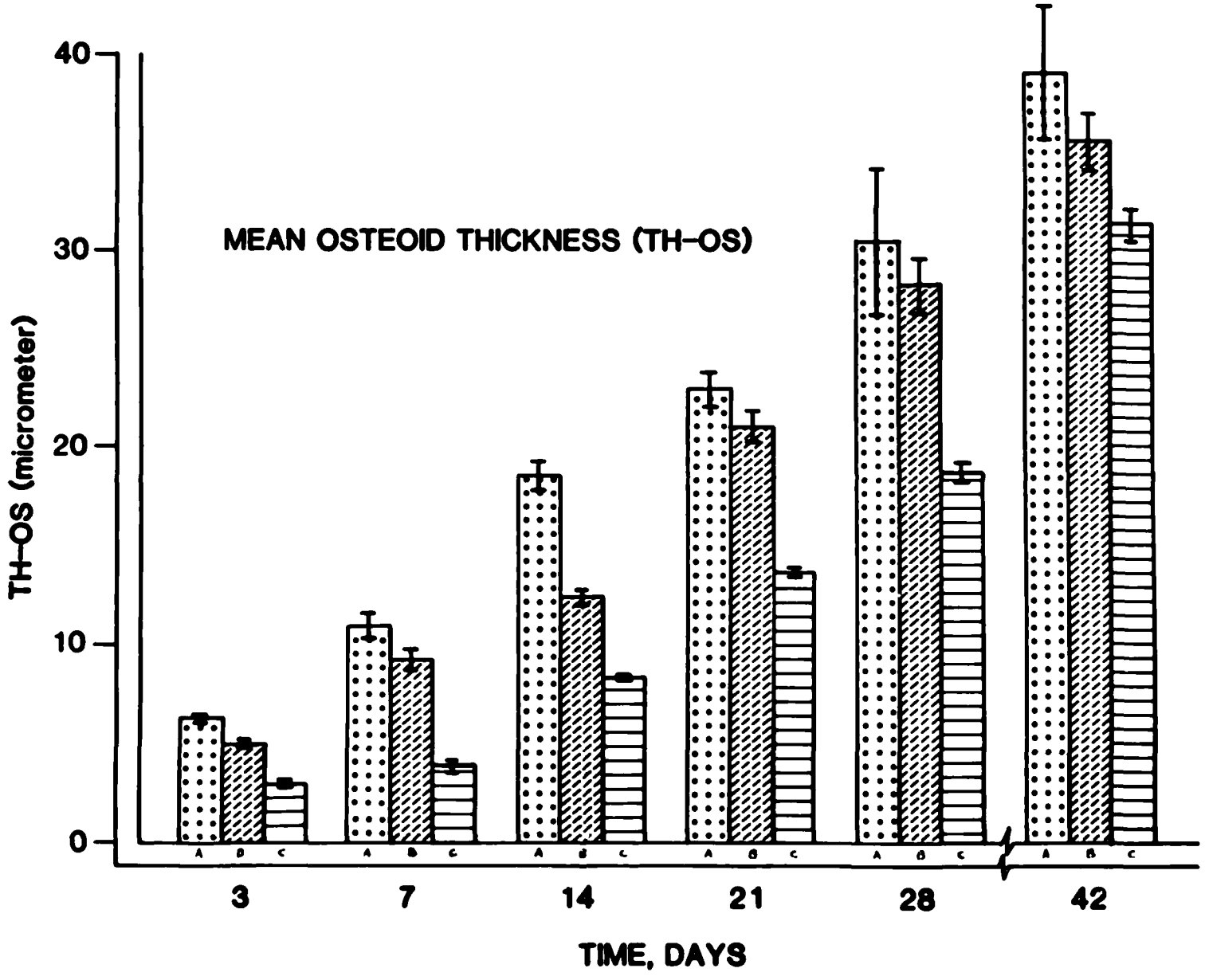
7576


VOLUMETRIC DENSITY OF MARROW (VV-MAR)
VV-MAR (mm³/cm³)
700
600
500
400
300
200
100
0
A B C
3
7
14
21
28
42
TIME, DAYS

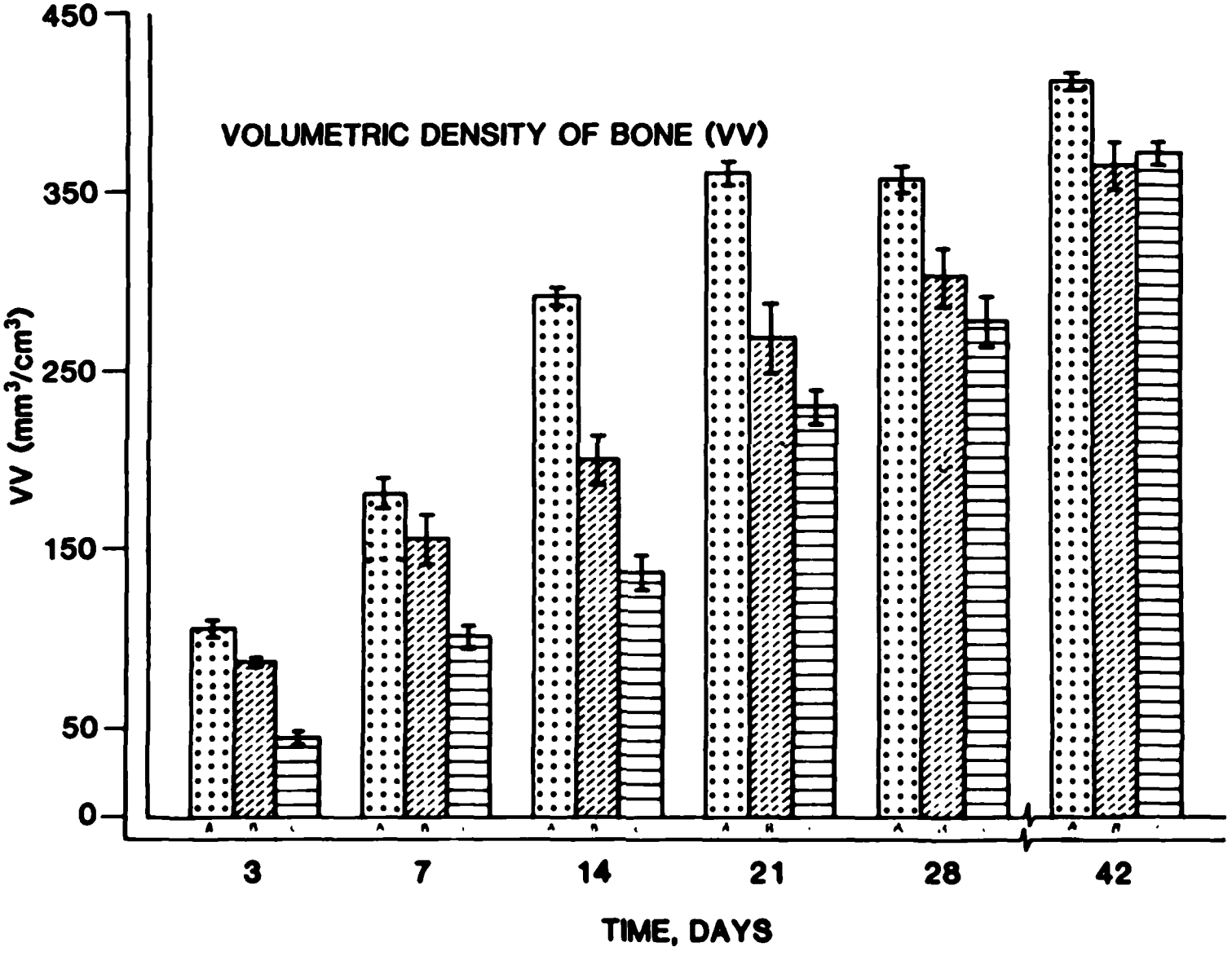
72403-9

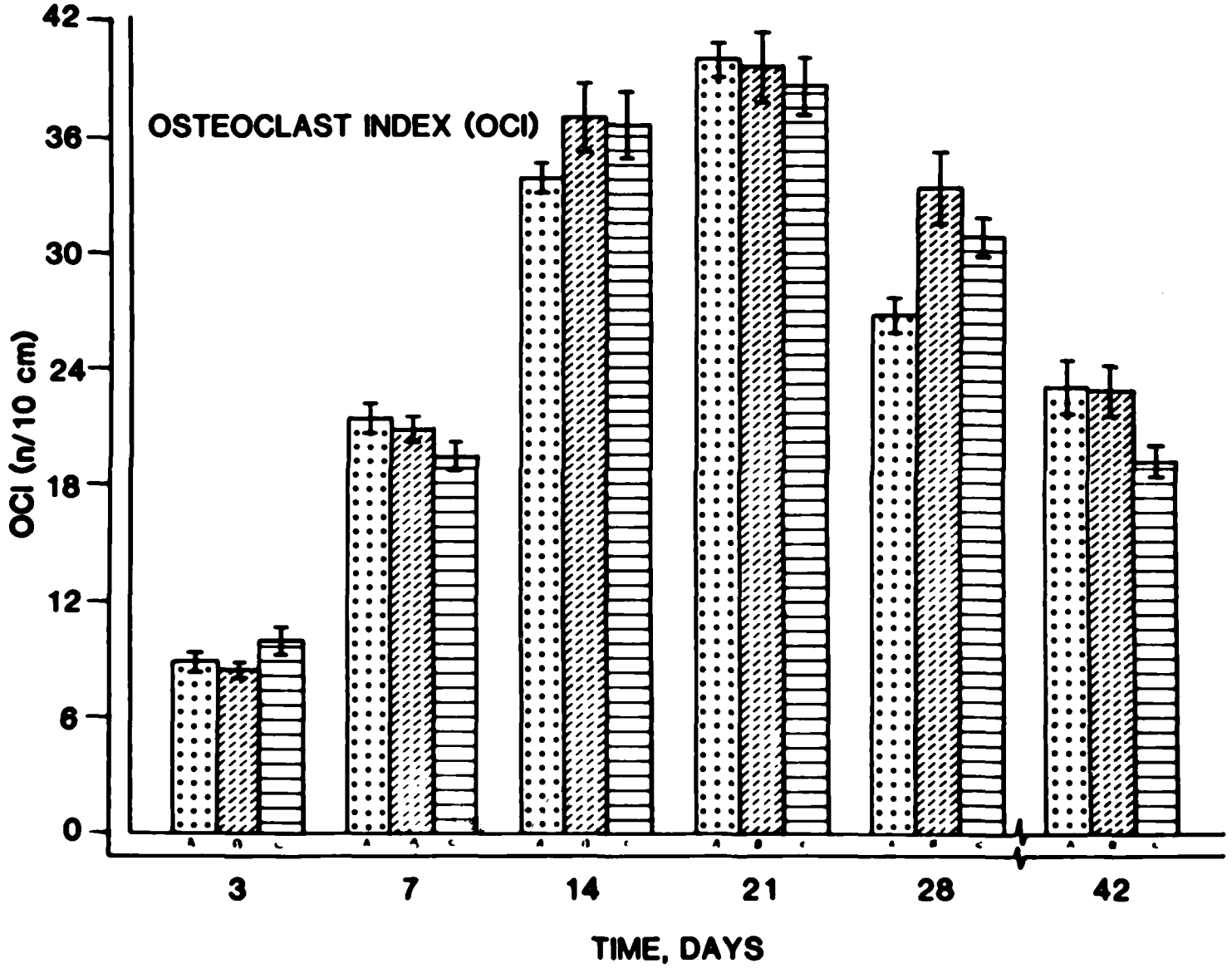

FRACTION OF
TRABECULAR SURFACE
EXHIBITING
RESORPTIVE LACUNAE
(L-TOT%)
L-TOT% (%)
14
12
10
8
6
4
2
0
A B C
3
7
14
21
28
42
TIME, DAYS

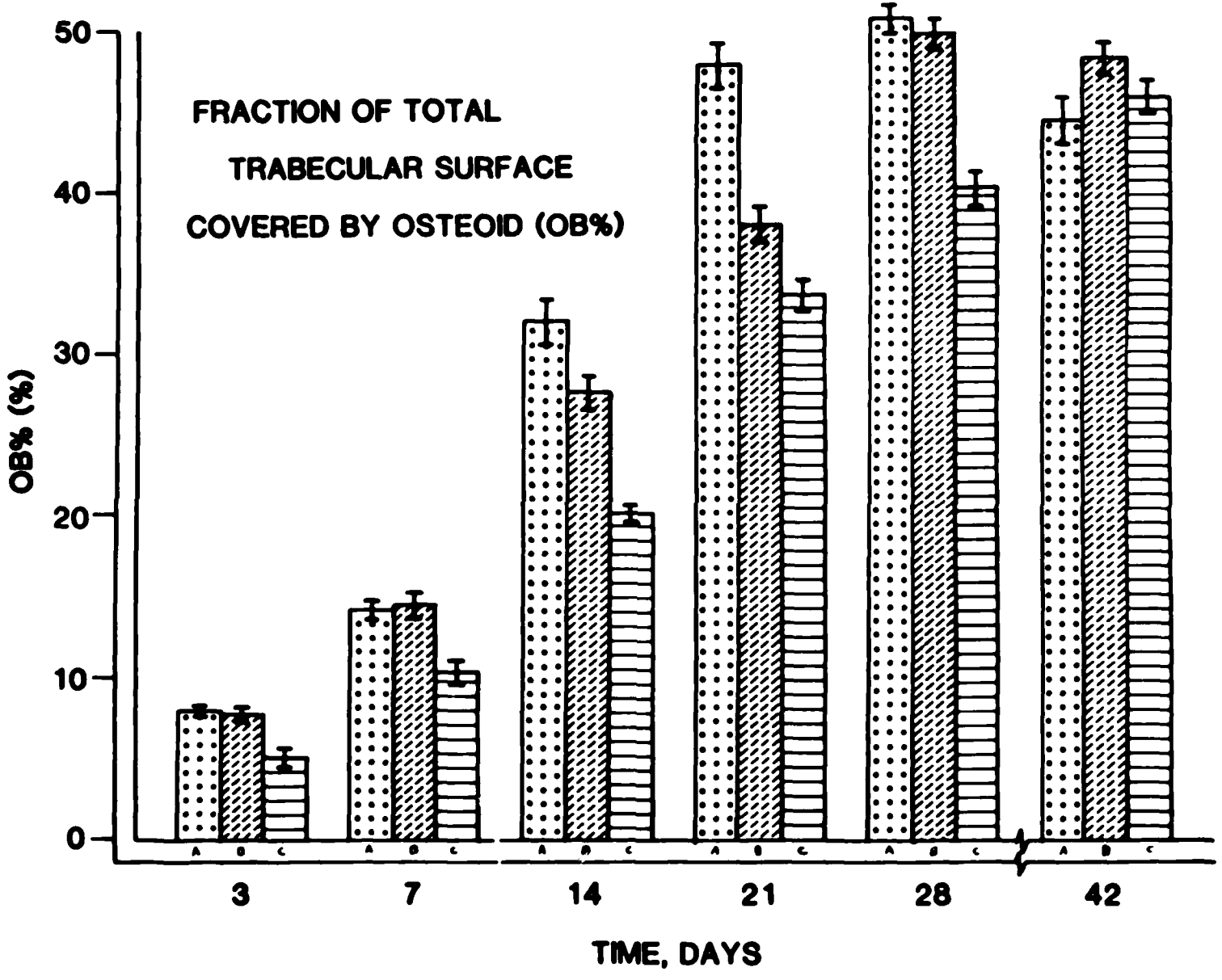
2240
72403-E


MEAN OSTEOID THICKNESS (TH-OS)
TH-OS (micrometer)
40
30
20
10
0
A B C
3
7
14
21
28
42
TIME, DAYS

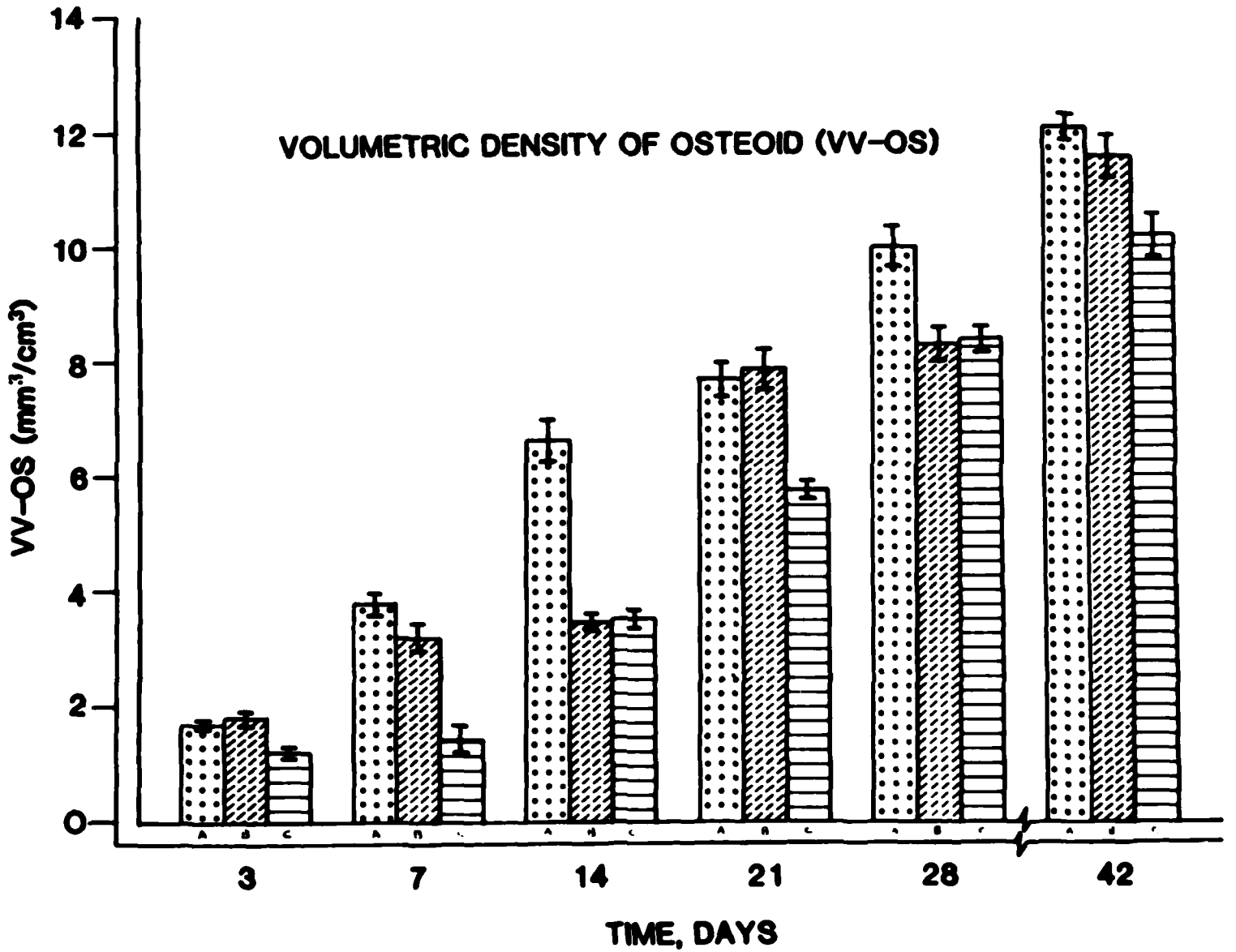
72403 - F

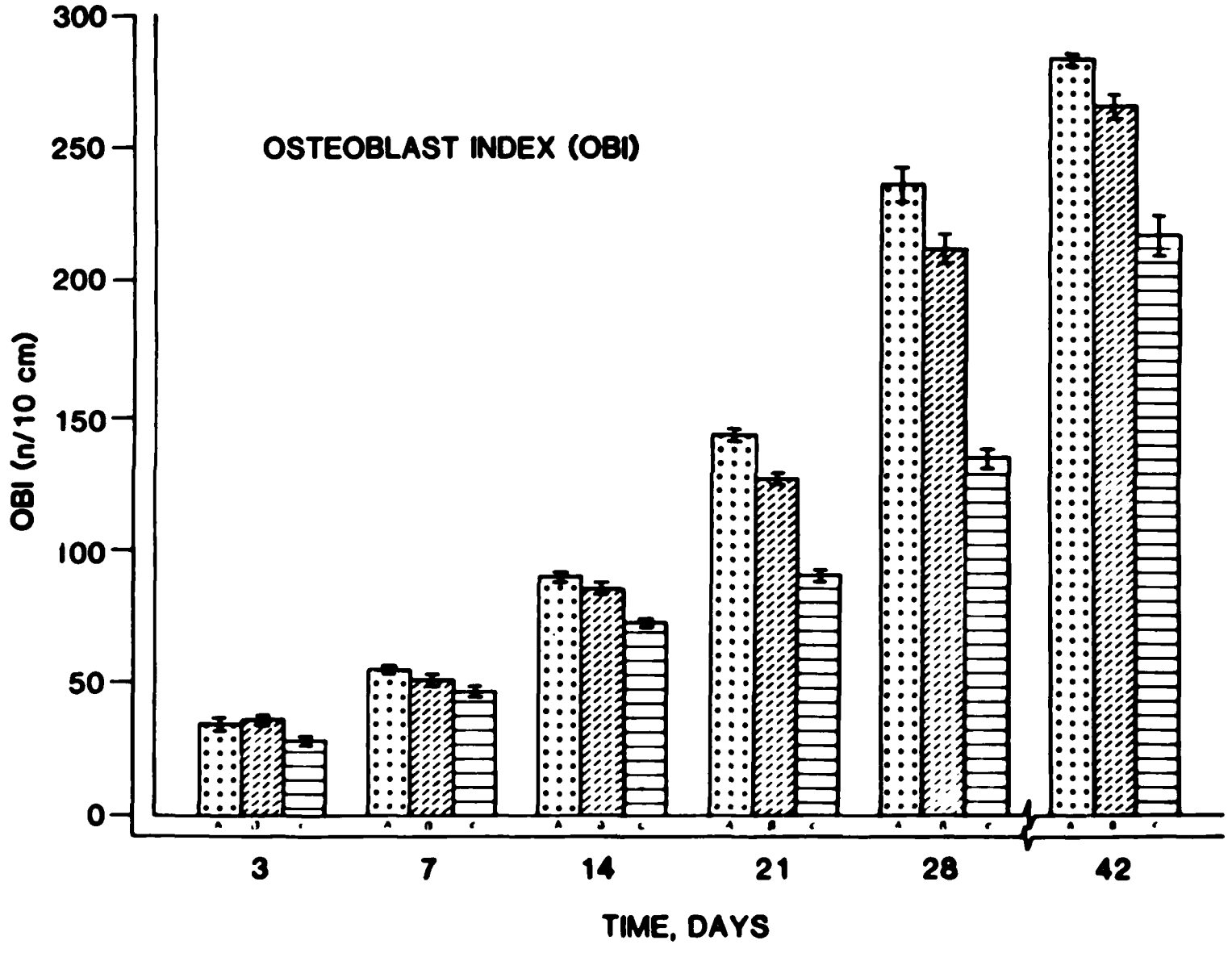

VOLUMETRIC DENSITY OF BONE (VV)
VV (mm³/cm³)
450
350
250
150
50
0
3
7
14
21
28
42
TIME, DAYS


OSTEOCLAST INDEX (OCI)
OCI (n/10 cm)
42
36
30
24
18
12
6
0
3
7
14
21
28
42
TIME, DAYS

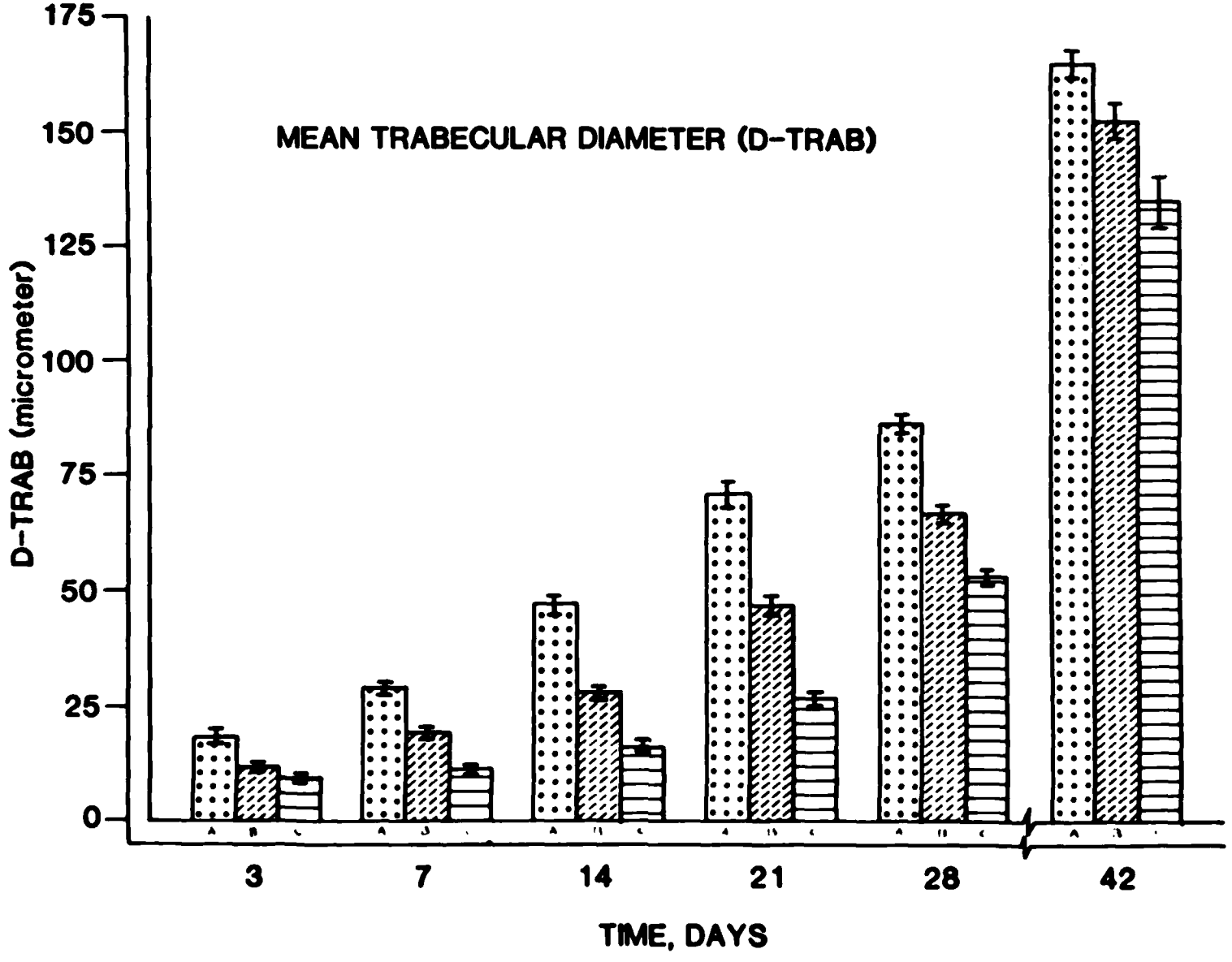

FRACTION OF TOTAL
TRABECULAR SURFACE
COVERED BY OSTEOID (OB%)
OB% (%)
50
40
30
20
10
0
A B C
3
7
14
21
28
42
TIME, DAYS

72403


VOLUMETRIC DENSITY OF OSTEOID (VV-OS)
VV-OS (mm³/cm³)
14
12
10
8
6
4
2
0
3
7
14
21
28
42
TIME, DAYS


OSTEOBLAST INDEX (OBI)
OBI (n/10 cm)
300
250
200
150
100
50
0
3
7
14
21
28
42
TIME, DAYS

72403-16


MEAN TRABECULAR DIAMETER (D-TRAB)
D-TRAB (micrometer)
175
150
125
100
75
50
25
0
3
7
14
21
28
42
TIME, DAYS

7243-0


DATE
ILME